PB85-193845

# Biochemical, Functional and Morphological Indicators of Neurotoxicity: Effects of Acute Administration of Trimethyltin to the Developing Rat

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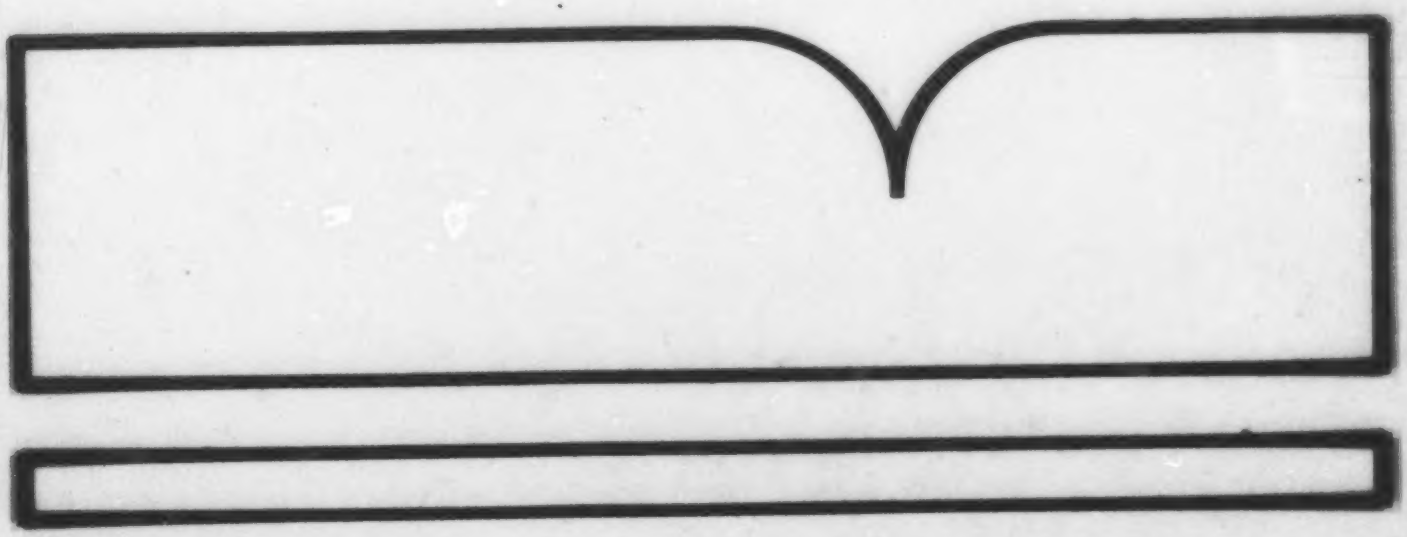
Research Triangle Park, NC

1984



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| 1. REPORT NO. | 2. | 3. R PB85-193845 |
|---|---|---|
| EPA/600/J-84/262 | | |

**4. TITLE AND SUBTITLE**
Biochemical, Functional and Morphological Indicators of Neurotoxicity: Effects of Acute Administration of Trimethyltin to the Developing Rat (Journal Version)

**5. REPORT DATE**
1984

**6. PERFORMING ORGANIZATION CODE**

**7. AUTHOR(S)**
Diane B. Miller and James P. O'Callaghan

**8. PERFORMING ORGANIZATION REPORT NO.**

**9. PERFORMING ORGANIZATION NAME AND ADDRESS**
Neurotoxicology Division
Health Effects Research Laboratory
US Environmental Protection Agency
Research Triangle Park, NC 27711

**10. PROGRAM ELEMENT NO.**
ACSL1A

**11. CONTRACT/GRANT NO.**

**12. SPONSORING AGENCY NAME AND ADDRESS**
Health Effects Research Laboratory
Office of Research and Development
US Environmental Protection Agency
Research Triangle Park, NC 27711

**13. TYPE OF REPORT AND PERIOD COVERED**

**14. SPONSORING AGENCY CODE**
EPA-600/11

**15. SUPPLEMENTARY NOTES**
Published In: The Journal of Pharmacology and Experimental Therapeutics 231(3): 744-751, Dec 1984

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**17. KEY WORDS AND DOCUMENT ANALYSIS**

| a. DESCRIPTORS | b. IDENTIFIERS/OPEN ENDED TERMS | c. COSATI Field Group |
|---|---|---|
| | | |

| 18. DISTRIBUTION STATEMENT | 19. SECURITY CLASS (This Report) | 21. NO. OF PAGES |
|---|---|---|
| RELEASE TO PUBLIC | UNCLASSIFIED | 9 |
| | 20. SECURITY CLASS (This page) UNCLASSIFIED | 22. PRICE |

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

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EPA/600/J-84/262
JOURNAL ARTICLE

Vol. 231, No. 3
*Printed in U.S.A.*

# Biochemical, Functional and Morphological Indicators of Neurotoxicity: Effects of Acute Administration of Trimethyltin to the Developing Rat[1]

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Accepted for publication August 27, 1984

**ABSTRACT**

The neurotoxic organometal, trimethyltin (TMT), was administered to rats on postnatal day (PND)5. Neurotoxicity was assessed throughout subsequent development using morphological, biochemical and functional endpoints. These consisted of brain weight measures and histology (morphology), assays of nervous system-specific proteins (biochemistry) and neurobehavioral indices of activity and learning (function). All three indices were affected. TMT caused dose-related decreases in brain weights at all ages examined, PND13, 22 and 66, with the hippocampus being the most severely reduced. Histological examination of the hippocampus revealed loss of pyramidal neurons in CA3 to CA4. Exposure to TMT was followed by dose- and age-dependent reductions in synapsin I, a neuron-specific phosphoprotein associated with synaptic vesicles; these effects of TMT were greater in hippocampus than in forebrain. TMT did not alter the concentration or protein composition of isolated myelin. The ontogeny of locomotion was altered in a dose- and time-dependent manner; TMT induced hypoactivity early in development (PND13) and hyperactivity by weaning (PND21); hyperactivity was also observed in the adult. Finally, TMT also affected learning ability throughout development. Deficits were observed in: 1) acquisition and retention of an instrumental alleyway response; 2) acquisition of a step-through passive avoidance response; and 3) radial-arm maze performance. These results demonstrate the use of a multi-endpoint, multi-timepoint strategy for the detection and characterization of neurotoxicity.

In the preceding paper (O'Callaghan and Miller, 1984) we reported that acute administration of TMT to the adult rat decreases neuron-specific phosphoproteins in the hippocampus, findings consistent with the effects of this compound on limbic system morphology. Acute administration of TMT to the developing rat also results in morphological alterations in the CNS (Ruppert *et al.*, 1983). We reasoned that these neuropathological effects of TMT, like those in the adult, might be accompanied by alterations in neuron or other cell-type specific proteins. Because the postnatal development of the rat CNS is characterized by neuronal maturation, glial proliferation and myelinogenesis (Dobbing, 1968), we chose to examine the effects of TMT on nervous system-specific proteins associated with these processes.

Severe functional deficits result from exposure of adult rats to TMT as indicated by neurobehavioral assessments of activity and learning (Dyer *et al.*, 1982) and it appears likely that similar alterations are associated with exposure to this compound early in development (see Ruppert *et al.*, 1983). Thus, in addition to morphological and biochemical measurements, we employed learning and activity paradigms tailored appropriately to the age and capabilities of the rat (see Miller, 1984) as indices of functional changes induced by early postnatal exposure to TMT. Our results indicate that the effects of TMT on CNS development are reflected in age-related alterations in morphology, nervous system-specific proteins and behavior.

## Materials and Methods

**Materials.** Materials were obtained as described in the preceding paper (O'Callaghan and Miller, 1984).

**Subjects.** Gravid Long-Evans rats (Blue Spruce Farms, Altamont, NY) were obtained on day 2 of gestation and housed individually in plastic tub cages (45 × 25 × 20 cm) with wood shavings as bedding. Pups born between 5:00 P.M. and 5:00 A.M. were considered to be born on the same day (PND0). On PND1 pups were reassigned to litters such that each dam received four male and four female pups and no more than one male and one female of her own offspring. Bedding was not changed from PND5 to PND13; from PND14 to 21 half of the soiled bedding was removed every 3rd day and replaced with clean

Received for publication March 12, 1984.

[1] This paper has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use. A preliminary report of this investigation appeared in Soc. Neurosci. Abstr. 9: 266, 1983.

**ABBREVIATIONS:** TMT, trimethyltin; CNS, central nervous system; PND, postnatal day; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RAM, radial-arm maze; TET, triethyltin.

bedding. At 21 days of age littermates were weaned, separated by sex and housed four per cage. Animals were maintained in a temperature (22°C ± 2)- and humidity (50% ± 10)-controlled colony room on a 12-hr light/12-hr dark cycle beginning at 6:00 A.M.

**Dosing.** Pups were injected (i.p.) on PND5 with 0.9% saline (0.0 mg/kg) or TMT hydroxide (5.0 or 6.0 mg/kg) dissolved in saline; this regimen was chosen because it results in minimal weight loss and mortality (see Ruppert *et al.*, 1983). Doses of TMT are expressed as the free base and were administered in a volume of 10 μl/g b.wt. All animals in a litter received the same dose and were foot tatooed with india ink for later identification. To control for the possibility of interactions between testing procedures animals were evaluated in only one behavioral paradigm. In addition, no more than two animals of each sex from a litter were used in a particular behavioral experiment; the remaining animals were removed for biochemical evaluations. Except where brains were prepared for histology, all animals were killed by decapitation.

**Brain and optic nerve dissection.** Immediately after decapitation whole brains were excised from the skull; the hippocampus, forebrain and cerebellum were removed by freehand dissection and wet weights were obtained. For synapsin I assays the forebrain was defined as the region remaining after removal of hippocampus and cerebellum from whole brain. For myelin analysis, the forebrain also included the hippocampus. Optic nerves (intraor[illegible]foramen to optic chiasm) were removed from one group of animals on PND22 and were immediately disrupted by sonification in 0.05% SDS. An aliquot of the optic nerve homogenate was taken for determination of total protein and samples were stored at −70°C before electrophoresis (see below). All dissections were performed on a refrigerated plate maintained at 0–4°C (Thermoelectrics Unlimited, Wilmington, DL). Samples from control and treated animals were always obtained at the same time.

**Histology.** On PND22, one group of animals that received TMT (6.0 mg/kg only) or saline was anesthetized (Chloropent, Fort Dodge Laboratories, Fort Dodge, IA) and perfused intracardially with 0.9% saline followed by buffered 10% Formalin. Whole brains were then carefully removed and stored in 10% Formalin for subsequent processing. Beginning at midline, sequential sagittal sections 10-μ thick were cut at 100-μ intervals from the left side of each brain. Sections were stained with cresyl violet.

**Radioimmunoassay of synapsin I.** On PND13, 22 and 66, the hippocampus and forebrain from one group of control and treated animals were dissected and homogenized immediately in 10 volumes of 1% SDS at 90°C. The relative specific activity of the neuron-specific phosphoprotein, synapsin I (De Camilli *et al.*, 1983a,b), was determined by detergent-based radioimmunoassay (Goelz *et al.*, 1981) as described in the previous paper (O'Callaghan and Miller, 1984). The data are presented on both a total and concentration basis as described previously (O'Callaghan and Miller, 1984).

**Myelin isolation.** On PND22, forebrains from one group of treated and control animals were dissected and homogenized immediately in 0.32 M sucrose. After an aliquot was removed for determination of total protein, myelin was isolated from the homogenate by the method of Norton and Poduslo (1973) using an SW-28 rotor in a Beckman L3-50 Ultracentrifuge maintained at 4°C. The isolated myelin was assayed for total protein before electrophoresis (see below).

**SDS-PAGE and microdensitometry.** Forebrain myelin and optic nerves were dissolved in an SDS-PAGE sample buffer (O'Callaghan *et al.*, 1983) and heated to 90–95°C for 2 min. The specific protein composition of these samples was then analyzed by SDS-PAGE. A detailed description of the SDS-PAGE procedures was given previously (O'Callaghan *et al.*, 1983; O'Callaghan and Miller, 1984) with the exception that the resolving gel contained 12% acrylamide. The concentration of small myelin basic protein, large myelin basic protein and proteolipid protein in samples of forebrain and optic nerve was determined by microdensitometry (LKB Ultrascan) of the dried gels. The areas under the peaks corresponding to each myelin protein are expressed as a percentage of total protein (see Toews *et al.*, 1980).

**Protein assays.** Protein was determined as described in the preceding paper (O'Callaghan and Miller, 1984).

**Assessment of locomotor activity.** On PND10, 13, 15, 17 and 21 the development of locomotor activity was determined in a Plexiglas open field (21 cm wide × 28 cm long × 25 cm high) with the wire-mesh floor demarcated into a 3 × 4 matrix of 7-cm squares. Individual subjects were observed for 3 min over home-cage litter with a count recorded each time the front paws crossed a line (see Miller, 1984).

In a separate set of animals, the generality of the effects of TMT on locomotor activity at weaning was measured in a photocell device (Motron Electronic Motility Meters, Motron Products, Stockholm, Sweden). Individual activity was determined only on PND21 in a 25-min session as described previously (see Miller, 1984).

**Assessment of learning ability.** An appetitive instrumental alleyway task in which home-cage litter and littermates serve as the reinforcer was used to determine the effect of TMT on learning ability during the preweaning period (PND14). In this task the animal is placed facing away from the goalbox, must learn to turn around and locomote down the alleyway to the goalbox in 120 sec or less. The procedure was as described by Miller (1984) with the following exceptions: 1) the floor of the alleyway was changed from Plexiglas to steel mesh to allow for the placement of home-cage litter beneath the entire apparatus during the acquisition period; 2) the acquisition criterion was changed from 5 to 2 consecutive trials and testing continued for only 5, rather than 15, trials during the performance phase; and 3) a 0.5-hr isolation period was interposed between the acquisition and performance phases so that memory or retention of the alleyway response could be determined.

The effect of TMT on learning ability during the postweaning, early juvenile period was determined in a second experiment by a passive-avoidance task. The passive-avoidance apparatus consisted of a V-shaped trough divided by a guillotine door into a small illuminated start box and a larger darkened box (Jarvik and Kopp, 1967). A trial was initiated by raising the guillotine door and a Coulbourn Instruments Constant Current Solid State Shocker (model E13-16) delivered a 0.5-mA shock each time the darkened chamber was entered. The AC shock was delivered to the feet of the animal through the stainless-steel panels forming the walls and floor of the darkened chamber. Testing began on PND22 and continued with one trial per day until two consecutive trials occurred in which the animal remained in the startbox for 5 min.

In a third experiment an automated RAM (see Miller *et al.*, 1982) was used to assess the effect of TMT on learning in the young adult. The maze consists of eight equidistantly spaced arms radiating from a central octagonal confinement area. A limited amount of food is available at the end of each arm and efficient performance requires the animal not to re-enter arms from which food has already been obtained. Photodiode pairs located in the center, arms and feeder cups allow the simultaneous determination of both accuracy and activity. Five-minute sessions (Monday–Friday) began on PND42 and continued for a total of 15 sessions past the session in which all arms were entered and all sucrose pellets (BioServe, Frenchtown, NJ) were eaten (see Miller, 1984 for details).

**Statistical analyses.** The Statistical Analysis System (SAS, 1982) was used for data analyses. When several measures (*e.g.*, body and brain weights) were collected on the same subject data were analyzed by a multivariate analysis of variance procedure (Morrison, 1967). Where multivariate analyses of variance were significant individual variables were analyzed by an analysis of variance or a repeated measure analysis of variance (Keppel, 1973). Sex of the subject was included as a variable in all analyses but was not found to interact with treatment; therefore, data from males and females were combined. In some instances dose-response relationships in the data were explored through the use of trend analyses (Winer, 1971). Duncan's Multiple Range Test was used to make mean comparisons. Details of the statistical evaluation for a particular measure appear in the figure or table legend.

TABLE 1

**Effect of acute postnatal administration of TMT on brain and body weights**

Each value represents the mean (±S.E.M.).

| Day | N | Dose | Body Wt. | Brain Wts. | Hippocampus Wts. | Cerebellum Wts. | Forebrain Wts. |
|---|---|---|---|---|---|---|---|
| | | mg/kg | | | g | | |
| 13 | 8 | 0.0 | 30.2 (±1.0) | 1.22 (±0.02) | 0.071 (±0.002) | 0.141 (±0.003) | 1.009 (±0.014) |
| 13 | 8 | 5.0 | 27.9 (±0.4) | 1.06 (±0.03)* | 0.051 (±0.003)* | 0.127 (±0.004)* | 0.880 (±0.240)* |
| 13 | 8 | 6.0 | 24.9 (±1.5)* | 0.88 (±0.09)*† | 0.033 (±0.007)*† | 0.118 (±0.006)*† | 0.671 (±0.060)*† |
| 22 | 8 | 0.0 | 49.2 (±2.8) | 1.48 (±0.01) | 0.082 (±0.003) | 0.199 (±0.004) | 1.188 (±0.011) |
| 22 | 8 | 5.0 | 45.3 (±1.1) | 1.23 (±0.02)* | 0.048 (±0.003)* | 0.181 (±0.004)* | 0.995 (±0.023)* |
| 22 | 8 | 6.0 | 43.8 (±2.3) | 1.20 (±0.05)*† | 0.041 (±0.003)* | 0.181 (±0.004)* | 0.905 (±0.032)*† |
| 66 | 8 | 0.0 | 268.9 (±19.3) | 1.90 (±0.03) | 0.113 (±0.004) | 0.281 (±0.004) | 1.508 (±0.020) |
| 66 | 8 | 5.0 | 255.8 (±21.5) | 1.62 (±0.03)* | 0.071 (±0.004)* | 0.265 (±0.004)* | 1.284 (±0.030)* |
| 66 | 8 | 6.0 | 247.3 (±17.5) | 1.47 (±0.03)*† | 0.053 (±0.004)*† | 0.252 (±0.004)* | 1.131 (±0.030)*† |

* Significantly different from the control, $P < .05$; † significantly different from 5.0 mg/kg, $P < .05$.

## Results

**Body and brain weights.** Administration of TMT on PND5 resulted in marked reductions in wet weight of whole brain, hippocampus, cerebellum and forebrain at PND13, 22 and 66 (table 1). Of the three brain regions examined, the hippocampal formation was the most affected; at a dose of 6.0 mg/kg, approximately a 50% reduction in the weight of this structure was observed at all three time points. In comparison to hippocampus, wet weights of cerebellum and forebrain were affected to a lesser degree at all time points and the greatest change was observed at PND13. In contrast to the effects of TMT on brain weight, body weights were minimally affected. Significant decrements were observed only in the high dose group during the preweaning period (PND13); no body weight changes were observed in the juvenile (PND22) or adult (PND66).

**Histology.** The effects of TMT (6.0 mg/kg, PND5) on the gross morphology of the hippocampus at PND22 are shown in figure 1. Representative sections obtained from dorsal hippocampus of a saline-treated subject (fig. 1A) and subjects moderately (fig. 1B) and severely (fig. 1C) affected by exposure to TMT are presented. The decreased hippocampal weights resulting from exposure to TMT (table 1) were reflected in a decreased size of this structure in all sections prepared from TMT-treated subjects; the range of effects of TMT on the size of the hippocampal formation are typified by the examples in figure 1. TMT also affected the cytoarchitecture of the hippocampus as evidenced by a loss of pyramidal cells in CA3 to CA4 and a diminished density of the remainder of the cell line. In contrast, no changes were observed in the gross morphology of cerebellum or forebrain after administration of TMT (data not shown).

**Radioimmunoassay of synapsin I.** The effects of TMT on the neuron-specific phosphoprotein, synapsin I, are presented in table 2. Administration of TMT on PND5 resulted in large dose-related decreases in total hippocampal synapsin I on PND13, 22 and 66. The decreases in total hippocampal synapsin I on PND13 and 22 were greater than the corresponding reductions in hippocampal wet weight as indicated by the 24 and 35% decrease in concentration of hippocampal synapsin I on PND13 and 22, respectively. By PND66 the concentration of hippocampal synapsin I did not differ significantly between saline and TMT-treated groups.

In comparison to the values obtained for hippocampus, radioimmunoassay values (total and concentration) for synapsin I in forebrain were less affected as a result of administration of TMT on PND5. Total synapsin I was reduced by as much as 45% on PND13 but by PND66 only a 21% reduction was observed at the 6.0-mg/kg dose. Dose-related decreases in the concentration of synapsin I were observed on PND13 and 22; however, these TMT-induced effects were never as large in magnitude as those observed for synapsin I concentration in hippocampus.

**CNS myelin.** The effects of TMT on indices of CNS myelination at PND22 are presented in table 3. Total protein and total myelin protein in forebrain as well as total protein in optic nerve were reduced as a function of the dose of TMT administered on PND5. However, because the decrease in isolated forebrain myelin was parallel to the decrease in forebrain weight, myelin concentration (milligrams of myelin protein per gram of forebrain) was not affected by TMT. In contrast, the decrease in total forebrain protein was greater than the decrease in forebrain weight as indicated by the slight reduction (approximately 5%) in forebrain protein concentration at the 6.0-mg/kg dose; a finding that indicates the possibility that nonmyelin protein components of forebrain were affected by TMT.

Representative electrophoretic profiles of isolated myelin and optic nerve protein are presented in figure 2. SDS-PAGE resolved the proteins characteristic of rat forebrain myelin: proteolipid protein ($M_r$ = 24 K), large basic protein ($M_r$ = 18 K) and the small myelin basic protein ($M_r$ = 14 K). These same proteins constitute a large percentage of total optic nerve protein (fig. 2., right side). The data obtained from densitometric scans (table 4) indicate that the protein composition of forebrain myelin and optic nerve was not affected by TMT.

**Locomotor activity.** Dose-dependent alterations in the development of locomotor activity were observed in the open field (fig. 3, left panel). The developmental curve for locomotion generally follows an inverted U-shaped function (see Bronstein *et al.*, 1974) similar to that observed in the saline-treated animals. TMT altered the shape of this curve; both TMT-treated groups were hypoactive relative to controls early in development (PND13) but became extremely hyperactive by weaning (PND21). The ontogeny of these toxicant-induced effects differed with respect to the dose of TMT administered on PND5. Thus, the time point chosen for activity assessment is a critical factor in determining both direction and magnitude of the effects of early postnatal exposure to TMT.

Figure 3 (right panel) illustrates that the hyperactivity found

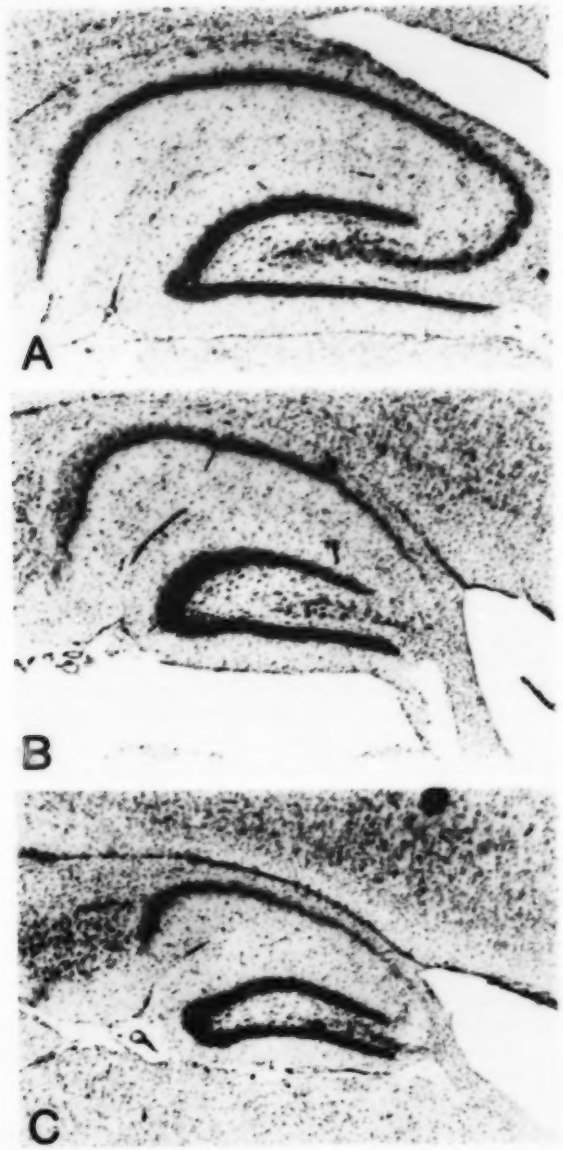


Fig. 1. Morphology of dorsal hippocampus on PND22 after acute administration of 6.0 mg/kg of TMT on PND5. Sections of brains from two TMT-treated animals are included to show moderate damage (B), severe damage (C) and control (A). The magnification is the same for all photomicrographs.

at weaning in the open field can be generalized to a photocell-based measure of activity, using a Motron device. The group treated with 6.0 mg/kg of TMT was hyperactive, as indicated by an increase in photocell interruptions relative to controls. TMT did not interfere with habituation to the apparatus, fewer responses were recorded during the final 5-min of testing relative to the first 5-min for all groups. A group exposed to 7.0 mg/kg of TMT on PND5 was included and showed almost a 400% increase in photocell interruptions relative to controls. However, this dose does cause some deaths before weaning.

**Learning ability.** Dose-related deficits in learning ability were apparent at all ages tested. In the preweaning alleyway task (table 5) animals treated with 6.0 mg/kg of TMT required

TABLE 2

**Effect of acute postnatal administration of TMT on synapsin I**

Each value represents the mean (±S.E.M.) of at least seven independent observations.

| Day | Dose | Hippocampus | | Forebrain | |
|---|---|---|---|---|---|
| | | Total | Conc. | Total | Conc. |
| | mg/kg | | | | |
| 13 | 0.0 | 1.00 (±0.13) | 1.00 (±0.10) | 1.00 (±0.10) | 1.00 (±0.10)* |
| 13 | 5.0 | 0.57 (±0.06)* | 0.81 (±0.08) | 0.75 (±0.02)* | 0.86 (±0.02) |
| 13 | 6.0 | 0.38 (±0.11)* | 0.76 (±0.05)* | 0.55 (±0.09)* | 0.81 (±0.06) |
| 22 | 0.0 | 1.00 (±0.10) | 1.00 (±0.07) | 1.00 (±0.09) | 1.00 (±0.08)* |
| 22 | 5.0 | 0.51 (±0.07)* | 0.90 (±0.09) | 0.78 (±0.08) | 0.94 (±0.09) |
| 22 | 6.0 | 0.33 (±0.05)* | 0.65 (±0.09)* | 0.62 (±0.06)* | 0.82 (±0.08) |
| 66 | 0.0 | 1.00 (±0.06) | 1.00 (±0.07)* | 1.00 (±0.08)* | 1.00 (±0.08)* |
| 66 | 5.0 | 0.60 (±0.07)* | 0.94 (±0.06) | 0.91 (±0.05) | 1.08 (±0.08) |
| 66 | 6.0 | 0.41 (±0.03)*† | 0.89 (±0.04) | 0.79 (±0.06) | 1.03 (±0.06) |

* $F(1,20)$ and P values (linear trend analysis) were 1.92, $P < .1806$ for concentration of synapsin I in hippocampus at PND66; 3.50, $P < .0753$ for total synapsin I in forebrain and 3.77, $P < .0665$; 2.30, $P < .1440$; 0.10, $P < .7538$ for concentration of synapsin I in forebrain at PND13, 22 and 66, respectively.

* Significantly different from 0.0 mg/kg at $P < .05$; † significantly different from 5.0 mg/kg at $P < .05$.

slightly more trials to exhibit a "homing" response. More importantly, these subjects were unable to retain the response over a 30-min delay. Only 40% of the animals receiving 6.0 mg/kg of TMT retained the response compared to 100% of the controls. Thirty percent of the group exposed to 5.0 mg/kg of TMT did not retain the homing response even though they acquired the response at the same rate as the saline-treated controls. TMT also increased the time required to reach the goalbox, a finding that may reflect the hypoactivity observed at this time (see fig. 3).

TMT also interfered with the acquisition of the passive avoidance response in a dose-related fashion (table 6); the group exposed to 6.0 mg/kg of TMT required almost twice as many days of training before they avoided as well as the controls. The group exposed to 5.0 mg/kg of TMT also required more days to avoid but this increase was not significant. Inasmuch as there were no differences in time to emerge from the startbox on the first day of training (table 6), the learning deficits probably cannot be attributed to treatment-induced increases in activity. Although it is possible that the deficiencies in passive avoidance are the result of treatment-related changes in shock sensitivity, this explanation also is not likely. All animals were observed to flinch or vocalize when shocked and in later testing with a nociceptive stimulus (hot-plate method, see O'Callaghan and Holtzman, 1975) the treated animals were found to be hyperalgesic (5.1, 4.9 and 3.0 sec to respond on a 51.5°C hot plate for the control and the groups exposed to 5.0 and 6.0 mg/kg of TMT, respectively [$F(2,29) = 5.48$, $P < .0096$].

Deficient RAM performance was also observed after TMT (fig. 4). Although no acquisition differences were found (*i.e.*, the number of days required to enter and eat in all alleys), differences were found in the accuracy with which the task was performed. The group exposed to 6.0 mg/kg of TMT always made more errors than controls (fig. 4, left panel), as well as selecting fewer correct arms in the first eight choices (6.3, 6.5 and 7.0; 6.2, 6.5 and 6.6; 5.6, 5.9 and 5.9 choices in the three consecutive 5-day blocks of testing for the control and the groups exposed to 5.0 and 6.0 mg/kg of TMT, respectively [$F(2,31) = 6.94$, $P < .0003$; 2.79, $P < .0769$; 16.36, $P < .0001$]

TABLE 3

**Effect of acute postnatal administration of TMT on indices of myelination at PND22**

Each value represents the mean (± S.E.M.) of at least six independent observations.

| Dose | Forebrain | | | | | Optic Nerve |
|---|---|---|---|---|---|---|
| | Wt. | Total | | Conc. | | Total |
| | | Protein | Myelin protein | Protein | Myelin protein | Protein |
| mg/kg | g | mg | mg | mg/g | mg/g | mg |
| 0.0 | 1.300 (±0.010) | 199.22 (±5.88) | 1.566 (±0.086) | 151.29 (±2.32) | 2.40 (±0.14) | 0.431 (±0.008) |
| 5.0 | 1.037 (±0.034)* | 175.03 (±9.96)* | 1.023 (±0.262)* | 150.42 (±1.77) | 2.29 (±0.13) | 0.400 (±0.010)* |
| 6.0 | 0.832 (±0.028)*† | 127.79 (±5.75)*† | 1.034 (±0.084)* | 144.84 (±1.94)* | 2.46 (±0.17) | 0.359 (±0.006)*† |

* Significantly different from control, $P < .05$; † significantly different from 5.0 mg/kg, $P < .05$.

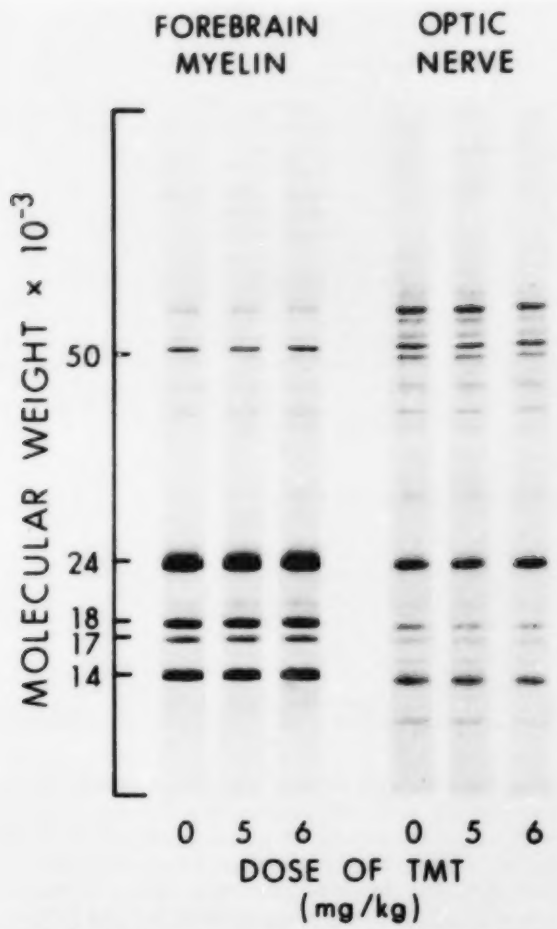


Fig. 2. Staining pattern of PND22 forebrain myelin and optic nerve proteins after resolution by SDS-PAGE. A total of 10 μg of protein were loaded on each lane.

for the treatment effect in the three respective blocks). No learning deficits were observed in the group exposed to 5.0 mg/kg of TMT but this and the group exposed to 6.0 mg/kg of TMT were hyperactive as indicated by an increase in photocell interruptions (fig. 4, right panel) and total arm selections (23.0, 22.6 and 23.5; 23.9, 28.3 and 29.9; 32.3, 35.1 and 35.2 arms in the three consecutive 5-day blocks of testing for the control and the groups exposed to 5.0 and 6.0 mg/kg of TMT, respectively [$F(2,31) = 7.64$, $P < .002$] for the overall treatment effect).

## Discussion

The neurotoxic organometal, TMT, interferes with brain development based on the following observations: 1) permanent weight deficits in forebrain, hippocampus and cerebellum; 2) loss of pyramidal cells in hippocampus; 3) age-related decreases in the neuron-specific phosphoprotein, synapsin I; and 4) alterations in activity and learning throughout the earlier portions of the lifespan of the animal. Data are discussed by category according to the three indices used to characterize the developmental neurotoxicity of TMT.

**Morphology.** Normal postnatal maturation of the rat brain requires the balanced interaction and timing of several critical processes, including cell division, migration and differentiation. Thus, it is not surprising that chemical and physical intervention during the early postnatal period alters many aspects of brain morphology, including brain weight (*e.g.*, Bayer *et al.*, 1973; Bohn, 1980; DeKosky *et al.*, 1982; Seress, 1978). In this study, TMT caused a large dose-related decrease in the weight of the hippocampus and less pronounced weight deficits in forebrain and cerebellum, findings that replicate the work of Ruppert *et al.* (1983) and extend it to several earlier postnatal ages. Whereas it is obvious that overall brain development was affected by the administration of TMT on PND5, our morphological findings are also indicative of an adult-like effect, *i.e.*, preferential involvement of the hippocampus. This notion is supported by three lines of evidence: 1) weight deficits in forebrain and cerebellum (expressed as a percentage of control) diminished with age whereas deficits in hippocampal weight remained constant; 2) damage to the hippocampus was characterized by loss of pyramidal cells [cells formed prenatally (Bayer, 1980)]; and 3) the pattern of pyramidal cell loss (CA3–CA4) resembled that seen in the adult (Brown *et al.*, 1979; O'Callaghan and Miller, 1984). Our morphological assessment of the developmental neurotoxicity of TMT was at best preliminary. Future studies would benefit from a detailed morphometric analysis of the neuronal damage as well as immunocytochemical characterization of the affected nervous system-specific proteins (see below).

**Nervous system-specific proteins.** We have proposed that toxicant-induced interference with nervous system development should be accompanied by alterations in nervous system-specific proteins (O'Callaghan and Miller, 1983). One such protein is synapsin I (formerly protein I), a neuron-specific phosphoprotein associated with synaptic vesicles (De Camilli *et al.*, 1983a,b). As synapsin I is apparently present in all nerve terminals (De Camilli *et al.*, 1983a) and its ontogeny coincides with synapse formation (Lohmann *et al.*, 1978), measurement of this protein may provide a procedure for determining the

**TABLE 4**

**Effect of acute postnatal administration of TMT on protein composition of forebrain and optic myelin at PND22**

Each value represents the mean (±S.E.M.) of at least six independent observations.

| Dose | % Total Protein | | | | | |
|---|---|---|---|---|---|---|
| | Forebrain | | | Optic nerve | | |
| | Small basic protein | Large basic protein | Proteolipid protein | Small basic protein | Large basic protein | Proteolipid protein |
| mg/kg | | | | | | |
| 0.0 | 17.85 (±0.85) | 15.22 (±0.81) | 31.89 (±1.47) | 14.29 (±1.25) | 4.64 (±0.41) | 22.21 (±1.95) |
| 5.0 | 18.58 (±1.11) | 15.89 (±0.88) | 30.65 (±1.52) | 12.00 (±1.10) | 4.41 (±0.47) | 19.31 (±2.72) |
| 6.0 | 18.60 (±1.31) | 16.21 (±0.86) | 30.36 (±1.49) | 14.56 (±1.90) | 4.99 (±0.51) | 15.82 (±2.41) |

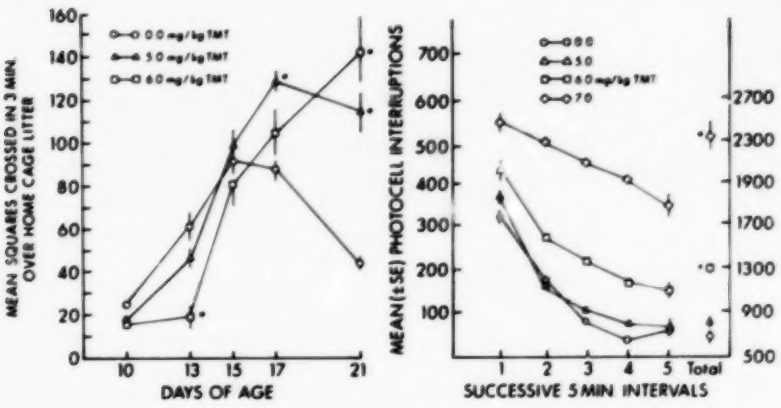
MEAN SQUARES CROSSED IN 3 MIN OVER HOME CAGE LITTER
DAYS OF AGE
MEAN (±SE) PHOTOCELL INTERRUPTIONS
SUCCESSIVE 5 MIN INTERVALS
Total

**Fig. 3.** Open-field activity over home-cage litter for the same animals at 10, 13, 15, 17 and 21 days of age as a function of dose (0.0, 5.0 or 6.0 mg/kg of TMT i.p.) on PND5. Repeated measure analysis of variance indicated an interaction between treatment and age [$F(8,124) = 9.63$, $P < .0001$]. Treatment effects were observed at PND10 [$F(2,31) = 4.10$, $P < .0264$]; PND13 [$F(2,31) = 12.44$, $P < .0001$]; PND17 [$F(2,31) = 8.22$, $P < .0014$]; PND21 [$F(2,31) = 13.76$, $P < .0001$]; but not PND15 [$F(2,31) = 0.78$, $P < .4685$]. Activity (photocell interruptions) at PND21 in the Motron (right panel) expressed as mean interruptions in the consecutive 5-min intervals of the 25-min session and mean total interruptions as a function of dose. A repeated measure analysis of variance showed an effect of treatment [$F(3,20) = 17.06$, $P < .0001$] and interval [$F(4,80) = 12.49$, $P < .0001$] but no interaction between these variables. *Significantly different from control at $P < .05$; +significantly different from 7.0 mg/kg of TMT-exposed group at $P < .05$.

**TABLE 5**

**Effect of acute postnatal administration of TMT on preweaning instrumental alleyway conditioning at PND14**

Each value represents the mean (±S.E.M.).

| Dose | N | Body wt. | No. of Trials to Homing Criterion | Time on Final Homing Trial | No. Retaining on First Retention Trial | Mean Time on Final Five Performance Trials |
|---|---|---|---|---|---|---|
| mg/kg | | g | | sec | | sec |
| 0.0 | 10 | 32.4 (±0.8) | 2.4 (±0.4) | 11.8 (±2.5) | 10/10 | 15.2 (±3.0) |
| 5.0 | 10 | 30.9 (±1.0) | 2.4 (±0.3) | 18.2 (±5.6) | 7/10* | 24.4 (±6.4) |
| 6.0 | 10 | 31.2 (±1.0) | 3.9 (±0.5)* | 37.1 (±6.0)* | 4/10** | 41.5 (±11.3)* |

* Significantly different from control, $P < .05$; * significantly different from control, $P < .05$; ** significantly different from control, $P < .002$ using test for significance of difference between two proportions (Bruning and Kintz, 1968).

**TABLE 6**

**Effect of acute postnatal administration of TMT on passive avoidance conditioning**

Each value represents the mean (±S.E.M.). Testing began at PND22.

| Dose | N | No. of Days to Avoidance Criterion | Time to emerge from the Start Box on Day 1 of Training |
|---|---|---|---|
| mg/kg | | | sec |
| 0.0 | 11 | 6.9 (±0.4) | 5.9 (±2.6) |
| 5.0 | 9 | 8.2 (±0.5) | 2.4 (±0.9) |
| 6.0 | 12 | 10.8 (±0.7)* | 11.1 (±3.2) |

* Significantly different from control, $P < .05$.

density of nerve terminals in neural tissue (Goelz *et al.*, 1981). In the present study we found that administration of TMT on PND5 resulted in large dose-related decreases in hippocampal synapsin I without causing comparable changes in forebrain synapsin I. These data indicate that the hippocampus was preferentially affected by exposure to TMT on PND5. Of particular significance is the fact that the concentration of synapsin I was reduced, findings suggestive of a reduction in synaptic density within the hippocampus. Several interpretations for these data can be advanced, among which are the following: 1) decrements in synapsin I concentrations may indicate that TMT interferes with events related to synaptogenesis and thereby delays synapse formation and 2) decrements in synapsin I concentration (PND13 and 22) may reflect, as they appear to in the adult (O'Callaghan and Miller, 1984), the extensive loss of pyramidal cells.

The effects of TMT on the concentration of hippocampal synapsin I during the preweaning (PND13) and juvenile (PND22) periods could not be extended to the adult (PND66). This apparent "recovery" in synapsin I concentration makes it tempting to speculate that the developing hippocampus may respond to TMT-induced injury with homotypic reinnervation and subsequent synapse formation, a phenomenon referred to as reactive synaptogenesis (Cotman and Nadler, 1978; Cotman *et al.*, 1981). Several recent observations support this hypothesis. For example, reactive synaptogenesis occurs more readily

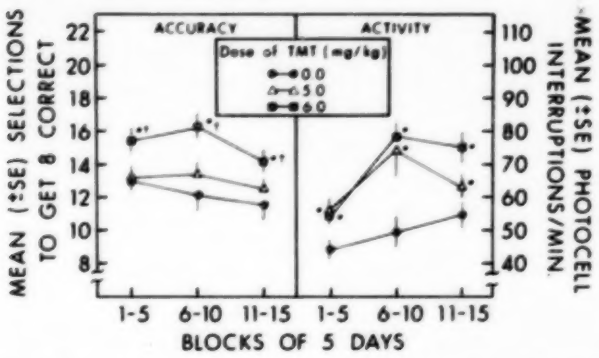


**Fig. 4.** Accuracy in the RAM expressed as mean total selections required to get eight correct arms for as a function of dose (0.0, 5.0 and 6.0 mg/kg i.p.) on PND5. Testing began at PND37. A repeated measure analysis of variance showed an effect of treatment [$F(2,31) = 9.74$, $P < .0005$] for total choices. The 6.0 mg/kg of TMT-exposed group consistently required more total choices to obtain all eight pellets compared to either the 5.0 or 0.0 mg/kg of TMT-exposed groups. Activity in the RAM is expressed as mean photodiode interruptions per minute. A repeated measure analysis of variance showed an effect of treatment [$F(2,31) = 4.10$, $P < .0263$] and block of testing [$F(2,62) = 10.31$, $P < .0001$]. TMT-treated groups were always more active than the saline-treated control but the activity of all groups increased as testing continued. *Significantly different from saline at $P < .05$.

in the immature animals than the adult (Gall and Lynch, 1980; Tsukahara, 1981; McWilliams and Lynch, 1983) and is especially prevalent in the hippocampal formation (Cotman and Nadler, 1978; Cotman *et al.*, 1981; Tsukahara, 1981). Although reactive synaptogenesis has been documented more extensively by reinnervation of dentate granule cells after lesions of the entorhinal cortex (Cotman *et al.*, 1981; Finger and Stein, 1982; Oswald and Vinsant, 1983), recent studies have shown that kainate-induced pyramidal cell loss in CA3 to CA4 is followed by gradual reinnervation, apparently by surviving homotypic fibers (Nadler *et al.*, 1980a,b). Thus, it is possible that reactive synaptogenesis in the hippocampus, like that observed with kainic acid, may result from exposure to TMT on PND5 and thus account for the apparent recovery in synapsin I concentration that occurred by PND66. Regardless of the changes associated with the recovery in hippocampal synapsin I, these events were not accompanied by a return to control values with respect to total synapsin I, hippocampal weight or neurobehavioral function.

Other nervous system-specific proteins may be used to monitor postnatal events associated with nervous system development. For example, oligodendroglia proliferation occurs just before the onset of myelination. Proteins specifically associated with myelin, *e.g.* myelin basic protein and proteolipid protein, become detectable at this time and increase in concentration as myelination proceeds (Norton, 1981). Thus, proteins specific to myelin may serve as a biochemical markers for events associated with the process of myelinogenesis. We have shown previously that TET, an organotin that is structurally related to TMT and known to damage rat myelin sheath (Jacobs *et al.*, 1977), appears to affect myelin after administration of TET on PND5 (O'Callaghan *et al.*, 1983). Although TMT and TET cause almost equivalent whole and regional brain weight decreases (O'Callaghan *et al.*, 1983; Miller, 1984), our present findings indicate that TMT does not affect myelin concentration or protein composition. The impact of TET on neuronal elements of either the developing or adult hippocampal formation has not been determined.

**Function.** Persistent alterations in activity often accompany toxic insult or injury to both developing and mature CNS (Bayer *et al.*, 1973; DeKosky *et al.*, 1982; Miller, 1984; Ruppert *et al.*, 1983; Walsh *et al.*, 1982). Here changes were observed in both the developing and mature animal and in a variety of monitoring devices. Our findings again reinforce the notion that the activity of an animal can serve as an index of CNS function (Grant, 1976; Reiter and MacPhail, 1982) and that the incorporation of home-cage cues into the test situation allows for the functional assessment of the preweaning rodent (see Miller, 1984; Smith and Spear, 1978). The effect of TMT on the development of locomotor activity presents an interesting paradox because the effect, hypoactivity, hyperactivity or no change in activity, is dependent on when in the developmental sequence the behavior is measured. The cerebellum and hippocampus both figure prominently in the expression of activity and interfering with the normal ontogeny of either structure by direct cytotoxicity or by preventing cell division and growth alters the characteristic pattern of locomotor development (Bayer *et al.*, 1973; Pelligrino and Altman, 1979). Thus, the ontogeny of TMT effects on preweaning locomotion may be due to damage in either of these brain areas. Whatever the cause, the presence of these differential effects does suggest the importance of including more than one time point when the development of a behavior is in question.

The weight deficits, loss in neurons and lowered synapsin I levels found in hippocampus provide support for the contention that TMT interferes with the development of this structure and it is reasonable to assume that hippocampal function should also be altered. In general, the behavioral deficits obtained, hyperactivity, poor retention of the preweaning learning task, slower passive avoidance acquisition and, in particular, the deficient RAM performance, are consistent with the notion that hippocampal function is altered (see Jarrard, 1976, 1980). However, to attribute the observed behavioral deficits solely to a dysfunction of the hippocampus, in particular, or the limbic system, in general, is unwarranted. Other brain areas are obviously affected by TMT. The forebrain is reduced in size and the possible alterations in the processing and integration of sensory information caused by the dysfunction of cortical areas cannot be dismissed as a potential contributing factor (see Dean and Key, 1981). No obvious functional deficits that can be attributed to compromised cerebellar function (*e.g.*, falls in the open-field or ataxia) accompanied the reduction in cerebellar size. However, the putative role of this structure in learned motor responses (see Thompson, 1983) as well as the ability of TMT to interfere with tasks reflecting cerebellar development (Ruppert *et al.*, 1983) suggests that the possible contribution of cerebellar dysfunction to the observed deficits cannot be overlooked.

**Acknowledgments**

The authors wish to thank Dr. Hany A. Makkawy and Drewry M. Vincent for excellent technical assistance, Christine Booth for expert preparation of the brain sections and Drs. Paul Greengard, Michael D. Browning and Linda J. Burdette for useful discussions.

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